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COMPARATIVE POLIOMYELITIS DIAGNOSIS IN TEST-TUBE TISSUE CULTURES AND IN PLASTIC CUPS

[Following is a translation of an article by Joachim Lehmann, Andreas Mahling, and Walter Hennessen in the German-language periodical Zeitschrift für Hygiene (Journal of Hygiene), 149, 1963, pages 114-121.]

Barski and Lépine [1] in 1954 described a micromethod for the serum neutralization test in which instead of "roller tubes" [the English expression appears here] plastic plates with regularly arranged depressions were used for the tissue culture. Through Rightsel et al. [9] and through Melnick and Opton [6] this idea was introduced into Salk et al.'s p_H color test [10], and at the same time more economical plastics were found that were inert with respect to tissue cultures.

Barski and Lépine's expensive "araldite B," which requires a great outlay for processing, was replaced with cheaper plastics from vinyl-mixture polymerizates [9] or from polystyrol [6] intended for one-time use. Lépine, Barski, and Cornefert [4] in 1956 also changed over with their microtest to the more advantageous PVC [polyvinylchloride] plastics, though they rejected the color test because of inaccuracies and retained the microscopic check on cytopathogenic effect. For one disadvantage detracts from the value of the color test on plastic plates: the defective and troublesome seal of the cell culture from the outside air. Adhesive films [1] or paraffin oil [6] or both together [9] are insecure sealants for liquids and are sensitive to shock, especially in shifting or transporting the cultures. After contact with air the nutrient medium becomes alkaline or unsterile, so that errors creep into the color test. In any case it tells against judging exclusively by the color change that finer distinctions such as beginning or late cell destruction or the peculiarities of the cytopathogenic effect of one species of virus can be determined only microscopically.

The method of work described by us below is likewise based

on the tested directions of the authors cited [1,4,6,9,10], all of whom were working toward a simplification of the test-tube culture test. We have made use of a new procedure for a surer seal of the openings in the plastic panel.

Material and Methods

Plastic cups. ([Note] Made by the firm of Fritz Kniese, Marburg/Lahn-Marbach.) -- Transparent 273x25 cm polyvinylchloride (PVC) sheets 0.25 mm thick, with eleven regularly spaced finger-stall-like depressions of 1.75 ml capacity; fitting cover sheets of the same material and the same size without depressions.

Sterilization of the plastic. -- Wash the cups and cover strips with distilled water, let stand in 70% undenatured alcohol two hours, dry in hot-air sterilized packing (paper bags, sheet aluminum boxes) at 37° C.

Tissue culture. -- 1. Primary culture [7,13]: Obtaining a cell suspension from monkey kidneys by trypsin dispersion, growing of "monolayer" cultures in rolled-edge tubes and on the bottom (240 cm^2) of a two-liter culture vessel (Fernbach flask), starting quantity for rolled-edge tubes 1 ml, for Fernbach flasks 100 ml of the cell suspension, growth phase to beginning of experiment 6-8 days.

2. Secondary culture [6]: Obtaining a cell suspension from the Fernbach flask after the completion of the growth phase. Procedure: After removal of the culture medium add 50 ml of 1:5000 dilute EDTA solution (EDTA = disodium salt of ethyl diaminotetraacetic acid); incubate at 37° C, agitating repeatedly until the cell layer is broken up (average time 45 minutes); centrifuge the cell yield 5 minutes at 900 g and decant liquid; take up the sediment in 15-25 ml of the nutrient medium (TCM [see note] + 3% calf serum). 1 ml of this cell suspension + 2 ml of a 0.1 mole aqueous solution of citric acid with 0.1% crystal violet for cell counting [11] in the Fuch-Rosenthal counting chamber. Set the cell suspension at 80,000 cells per milliliter by dilution with nutrient medium. Antibiotic content: 25 γ neomycin, 25 γ streptomycin, and 25 units of penicillin G per milliliter.

[Note] Synthetic nutrient medium for tissue cultures after Morgan, Morton, and Parker, hereinafter called TCM.

Virus. -- Polio virus of Type I Mahoney, Type II MEF$_1$, and Type III Saukett. Thin the virus suspension in geometric series on the base 10 with Hank's solution and with TCM as dilution media. Inoculate four (or six or ten) tube cultures and as many cups with the dilution stages prepared from the virus suspension. Amount, 1 ml per tube and 0.25 ml per cup, followed by addition of 0.25 ml of the secondary suspension (20,000 cells) in each cup. Tissue degeneration control by means of undisturbed control cultures.

Serum. -- Anti-polio myelitis sera from guinea pigs and from rabbits with neutralizing antibodies against one or against three polio virus types. Dilute the sera in geometric series on the base 4 -- or 2 -- with TCM as diluent. Mix each dilution stage in the ratio 1:1 with homologous virus suspensions in the concentration of 100 ID_{50}/0.25 ml (exact concentration, with simultaneous control titration). Then pour in 0.5 ml of the virus-serum mixture, followed by 1 ml of nutrient medium (TCM) per tube culture and 0.25 ml of the mixture per cup followed by 0.25 ml of the secondary cell suspension (20,000 cells). Four tube cultures or cups per dilution stage. Control cultures, first to observe the tissue degeneration and second for toxicity control.

Inoculation technique. -- Testtube inoculation as usual [13]. Cup inoculation in an inoculation chamber in which most of the germs have been killed by ultraviolet radiation. Remove the strips containing the cups from the sterile packing and lay them in the pressure block of the welding device (see below). Put in the test material and the cell suspension one after the other (order immaterial), filling one cup per strip as a control with 0.25 ml of TCM instead of the test material. Lay on the sterile cover sheets, remove from the inoculation chamber, and seal the cups by welding.

Plastic Welding Device. (See note and Figure 1.) -- Mode of operation: The sheets of plastic to be welded are pressed between press block and press bar and heated for a short time with a heating element laid over them [12].

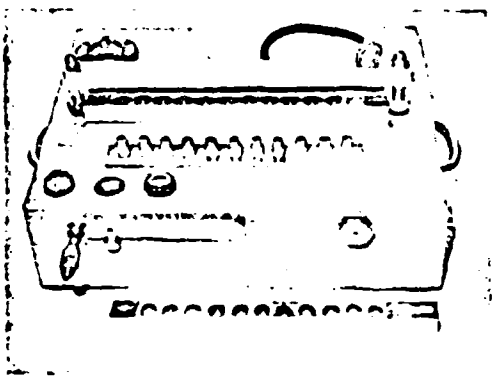

Fig. 1. Plastic Welding Device. (Press block to lay panels in in the foreground.)
[Note] Made by the firm of Fritz Kniese, Marburg/Lahn-Marbach.

Evaluation. -- Microscopic examination of the tubes and cups incubated at 37° C, three and seven days after the beginning of the experiment. Titration by Reed and Muench's method [8] on the seventh day without taking account of the color change.

Results

A. The same virus strain suspension (polio virus type III, Saukett) was titrated on 13 days of the experiment and the infection titre obtained comparatively for tubes and cups. (For results see Table 1.)

In the titre computation the different amounts per tube (1 ml of virus suspension) and per cup (0.25 ml of virus suspension) must be taken into account; the virus titre obtained by the cup method must be multiplied by a factor of 4 (or the log 4 = 0.6021 added to the

Table 1

Experiment No.	Cup Method (virus suspension together with secondary cell suspension in plastic cups) Virus Titre: $x_{iN} = (\log ID_{50})_{iN/ml}$	Tube Method (virus suspension on matured primary tissue in rolled-edge tubes) Virus Titre: $x_{iR} = (\log ID_{50})_{iR/ml}$
1	6.10	7.25
2	6.60	6.60
3	7.10	7.00
4	6.31	6.88
5	7.00	6.88
6	6.75	6.75
7	6.60	7.00
8	7.31	6.60
9	7.10	6.50
10	6.35	7.36
11	6.10	6.75
12	tissue degenerated	6.60
13	6.35	6.50

Table 2

	Cup Method	Testtube Method
$\bar{x}$	6.6392	6.8708
$s^2 = \frac{\sum (x_i - \bar{x})^2}{N-1}$	0.17070	0.07569
N	12	13
$\pm s$	0.4132	0.2751
$\pm t_{95\%} \cdot s$	0.9095	0.5994

$\bar{x} = \overline{\lg ID_{50}}$ = average of the log of the virus titre
s^2 = variance of the titre
N = number of experiments
$\pm s$ = standard deviation
$\pm t_{95\%} \cdot s$ = 95% confidence range of an individual measurement
$t_{95\%}$ = "student function" for N - 1 degrees of freedom

logarithm of the virus titre) when we compare with the testtube titre.

The evaluation of the titres is summarized in Table 2.

From Table 2 the following conclusions can be drawn:

1. The variances of the virus titres by the two methods prove significantly different by Fisher's F test [3]; the cup method has a wider scatter range than the testtube method. The 95% confidence ranges are considered a measure of this.

2. The average values differ only accidentally from each other, and it may be said that the two methods lead to the same results.

Table 3

Experiment No.	Cup Method y_{iN} = (log of reciprocal of serum titre)$_{iN}$	Testtube Method y_{iR} = (log of reciprocal of serum titre)$_{iR}$
1	3.21	2.81
2	2.61	2.71
3	3.10	2.81
4	3.11	2.71
5	2.81	2.71
6	2.61	2.71
7	2.41	2.71
8	2.71	2.61
9	2.81	3.10
10	2.21	2.21
11	3.31	2.87
12	tissue degenerated	2.71
13	2.71	2.71

Table 4

	Cup Method	Testtube Method
$\bar{y}$	2.8008	2.7215
$s^2 = \frac{\sum (y_i - \bar{y})^2}{N-1}$	0.1094	0.0380
N	12	13
$\pm s$	0.3308	0.1949
$\pm t_{0.05} \cdot s$	0.7208	0.4247

$\bar{y}$ = log of reciprocal serum titre = average of the logarithmic serum titre. For other explanations see Table 2.

Because of the difference in the variance, strict statistical testing by Cochran and Cox's method [2] was used.

B. With the same experimental procedure thirteen serum neutralization tests were done with the same serum (rabbit anti-type III hyperimmune serum) against the above-mentioned polio virus type III. (For results see Table 3.)

The results may be summarized as shown in Table 4.

As in the case of the virus titres of Table 2, the serum titres of the cup method show a greater variance in Fisher's F test than the titres of the testtube method. Here too the average values prove to differ from each other only by chance.

Cochran and Cox's method [2] was again used for statistical evaluation.

C. With 21 different guinea pig mixed sera from immunization experiments neutralization tests were done against all three polio virus types. For results see Table 5.

Table 5

Guinea Pig Mixed Serum No.	Polio Virus Type	Num. Recip. Serum Titre		Titre Difference $d_i = y_i - x_i$
		Cup Method y_i	Testtube Method x_i	
1	I Mahoney	32	16	+ 16
2		323	128	+195
3		128	81	+ 47
6		128	102	+ 26
7		tissue degenerated	—	—
8		25	25	± 0
9		46	64	- 18
10		40	32	+ 8
11		256	128	+128
12		256	64	+192
13		102	128	- 26
14		128	128	± 0
15		25	16	+ 9
16		ε	81	+ 6
17		—	tissue unsterile	—
18		185	406	-221
19		161	128	+ 33
20		256	161	+ 95
21		161	406	-245
1	II MEF$_1$	tissue degenerated	—	—
2		51	161	-110
4		185	46	+139
6		81	40	+ 41
7		13	46	- 33
8		16	32	- 16
9		64	25	+ 39
10		12	21	- 9
11		256	128	+128
12		256	161	+ 95
13		128	161	- 33
14		32	32	± 0
15		13	22	- 9
16		64	256	-192
17		16	22	- 6
18		64	128	- 64
19		32	81	- 49
20		406	406	± 0
21		161	161	± 0

Table 5 (continued)

Guinea Pig Mixed Serum No.	Polio Virus Type	Num. Recip. Serum Titre		Titre Difference $d_i = y_i - x_i$
		Cup Method y_i	Testtube Method x_i	
1	III Saukett	16	88	- 72
2		46	46	± 0
5		83	26	+ 57
6		10	33	- 23
7		tissue degenerated	—	—
8		tissue degenerated	—	—
9		16	25	- 9
10		tissue degenerated	—	—
11		32	25	+ 7
12		6	8	+ 2
13		64	40	+ 24
14		8	6	+ 2
15		12	10	+ 2
16		32	32	± 0
17		32	16	+ 16
18		32	25	+ 7
19		40	25	+ 15
20		40	102	- 62
21		406	128	+278
		5064	4658	+406

Total: Sy_i = 5,064 Sy_i^2 = 1,043,138

Total: Sx_i = 4,658 Sy_i^2 = 898,684

Total: Sd_i = 406 Sd_i^2 = 411,938 $Sy_i \cdot x_i$ = 764,942

Between the y_i's and the x_i's there is a correlation whose definiteness (Bestimmtheit) B can be calculated from the sums of the squares by the following equation:

$$B = \frac{(Sy_i \cdot x_i)^2}{Sx_i^2 \cdot Sy_i^2} *$$

$$B = \frac{1043138^2}{898684 \cdot 764942}$$

$$B = 0{,}6242$$

*Between the definiteness B and the coefficient of correlation r in this case the relationship $r = +\sqrt{B}$ obtains.

A linear regression of the form

$$y = b \cdot x \qquad b = \text{constant}$$

is assumed. The number of degrees of freedom is 51 - 1 = 50.

The conv. factor must be regarded as strongly confirmed if B becomes greater than 0.1301 [5]. For the average difference between the two methods we find $\bar{d} = \frac{406}{51} = +7{,}9608$ titre units. If we compare this average value to its simple standard deviation

$$s = \pm\sqrt{\frac{S d_i^2}{n(n-1)}} = \pm 12{,}66$$

we find that it differs from zero only by chance, for the difference average difference minus simple standard deviation embraces the value zero. It is thus established that the two methods yield the same titre values.

Discussion

The reason for comparing the two test methods was the simplification offered by the use of plastic cups. A considerable saving in labor can be achieved by not having to grow testtube cultures. Expenditures for materials can also be reduced, even when the steady use of plastic panels is taken into account. Lastly, there is a saving in space that is obvious from comparison of the size of testtubes and plastic cups.

The variances in logarithmic virus titres and serum titres in Table 1 and Table 3 respectively may be largely explained by pipetting errors. Since the amount put into each cup was less than the amount per testtube and since the same size ratio could not be maintained in pipettes as in container volumes (slow run-through time with small pipettes!) the relative error in volume, and consequently the absolute error in logarithmic titre, was greater in the cup method than in the testtube method. We considered this disadvantage less than the advantage of time saved in pipetting. The definiteness of 62.42% which can be computed from the data in Table 5 corresponds to an indefiniteness of 100 - 62.42 = 37.58%. It is conceivable that this remaining indefiniteness can be explained not by accidental errors, e.g. in pipetting, but also by schematic differences between the series of experiments. Probably we should consider differences in the ratio of the original concentration of free infectious virus particles to the concentration of cells.

Summary

With the poliomyelitis virus as an example, the virus concentration and neutralization by antisera were determined by two methods:

1. In the traditional testtube cultures, and
2. In plastic cups with subcultures.

The results were statistically compared with each other. It was found that the titre averages for the two methods differ only by chance, but that the cup method has a greater variance. It may still be said that the cup method is just as usable for virus and serum diagnosis of poliomyelitis as the testtube method.

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